

Active endocannabinoids are secreted on extracellular membrane vesicles

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Abstract

Endocannabinoids primarily influence neuronal synaptic communication within the nervous system. To exert their function, endocannabinoids need to travel across the intercellular space. However, how hydrophobic endocannabinoids cross cell membranes and move extracellularly remains an unresolved problem. Here, we show that endocannabinoids are secreted through extracellular membrane vesicles produced by microglial cells. We demonstrate that microglial extracellular vesicles carry on their surface *N*-arachidonylethanolamine (AEA), which is able to stimulate type-1 cannabinoid receptors (CB₁), and inhibit presynaptic transmission, in target GABAergic neurons. This is the first demonstration of a functional role of extracellular vesicular transport of endocannabinoids.

Keywords anandamide; CB₁ receptor; extracellular vesicle; GABA release; microglia-neuron signaling

Subject Categories Membrane & Intracellular Transport; Neuroscience

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Introduction

Endocannabinoids (eCBs) are lipid messengers which potently modulate synaptic function [1]. Their impact on synaptic transmission is widespread and diverse: Short-term forms of plasticity induced by eCBs have been described in numerous brain areas, while eCB-mediated modulation of long-term plasticity has implicated these bioactive lipids in learning and memory [2,3]. Since eCB-binding receptors are localized more prominently on inhibitory than excitatory terminals, eCB signaling is widely thought to be involved in regulating over-excitability and in promoting synaptic

homeostasis. In addition to modulating the activity of mature synapses, eCBs have been implicated in synapse formation and neurogenesis [4].

Retrograde signaling is the principal mode by which eCBs modulate synaptic function. *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), the most active eCBs as yet discovered, are synthesized in the postsynaptic compartment of neurons and act retrogradely to inhibit GABA or glutamate release from presynaptic terminals; this process is mediated by activation of type-1 cannabinoid (CB₁) receptor [3,5]. However, there is also evidence suggesting that eCBs indirectly signal at the synapse via glial cells by triggering gliotransmission [6] and that glial cells directly produce eCBs [7]. Indeed, microglia release eCBs [8–10] and produce *in vitro* 20-fold higher amounts than neurons or astrocytes, likely representing the main source of these substances in the inflamed brain [11]. Microglia also respond to eCBs through functional type-1 (CB₁) and type-2 (CB₂) cannabinoid receptors, which likely regulate microglia behavior and phenotype [11].

Microglia release different regulators of synaptic activity (among others BDNF and TNF- α), which not only modulate synaptic transmission in response to injury and inflammation, but also play a central role in homeostatic synaptic plasticity and in neuronal network development [12,13]. Although the central role of eCB signaling in neuronal function is widely accepted, whether eCBs produced by microglia modulate synaptic transmission remains yet to be explored [1]. In this context, it is not yet clear how hydrophobic eCBs may travel across the extracellular space to reach target neurons [5,14].

Reactive microglia release into the pericellular space extracellular vesicles (EVs), which represent ideal vehicles for the transport of hydrophobic signaling molecules across the nervous system [15]. As in most cell types, EVs emanate through the outward blebbing of the microglial surface and are called ectosomes or shed microvesicles (MVs). Alternatively, EVs form inside multivesicular bodies (MVBs) in the endosomal system and are secreted as exosomes upon MVB fusion with the plasma membrane [16–18].

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Some components of the eCB system, including CB₁ receptors, have been previously described in MVs shed from microglia [17]. Furthermore, *in vitro* production and release of 2-AG from microglia is known to increase upon cell exposure to ATP [9], which represents the main stimulus driving MVs release from the microglial surface through activation of the ATP receptor P2X₇ [19]. Against this background, here we investigated whether eCBs may be released by microglia in association with EVs and whether they may influence neuronal synaptic communication.

Results

AEA is enriched in MVs and exosomes released by microglial cells

First, we measured eCB content in a mixed population of EVs, produced by murine N9 microglia or primary rat microglia exposed to 1 mM ATP for 1 h and collected from the cell supernatant by ultracentrifugation at 100,000 g after pre-clearing from cells and debris (modified from 17). This condition mimics an inflammatory

context and induces shedding of MVs from the microglial surface, but also allows extracellular accumulation of exosomes, constitutively released from the endocytic compartment [17]. Similar production of MVs relative to exosomes, released from primary microglia, was indeed revealed by: (i) nanoparticle tracking analysis of MV- and exosome-enriched pellets, collected by differential centrifugation at 10,000 g and 100,000 g, respectively (Fig 1A), (ii) quantification of the protein content of the two vesicle fractions (Fig 1B), and (iii) Western blotting of MV- and exosome-enriched fractions for the general EV marker Tsg101 [20,21], for flotillin, which preferentially stained MVs, and alix, a vesicular marker enriched in exosomes (Fig 1C). We found detectable amounts of AEA in mixed EV population and in corresponding donor cells (Fig 1D). Interestingly, AEA content was up to ~2,900-fold higher in EVs compared to parental N9 cells while an enrichment by ~120-fold was detected in EVs produced from primary microglia (Fig 1D). Conversely, 2-AG was below the limit of detection (LOD = 0.2 pmol/mg of protein) in all samples tested. To determine which type of EVs actually carries AEA, we next analyzed the eCB content in MV- and exosome-enriched fractions. This analysis

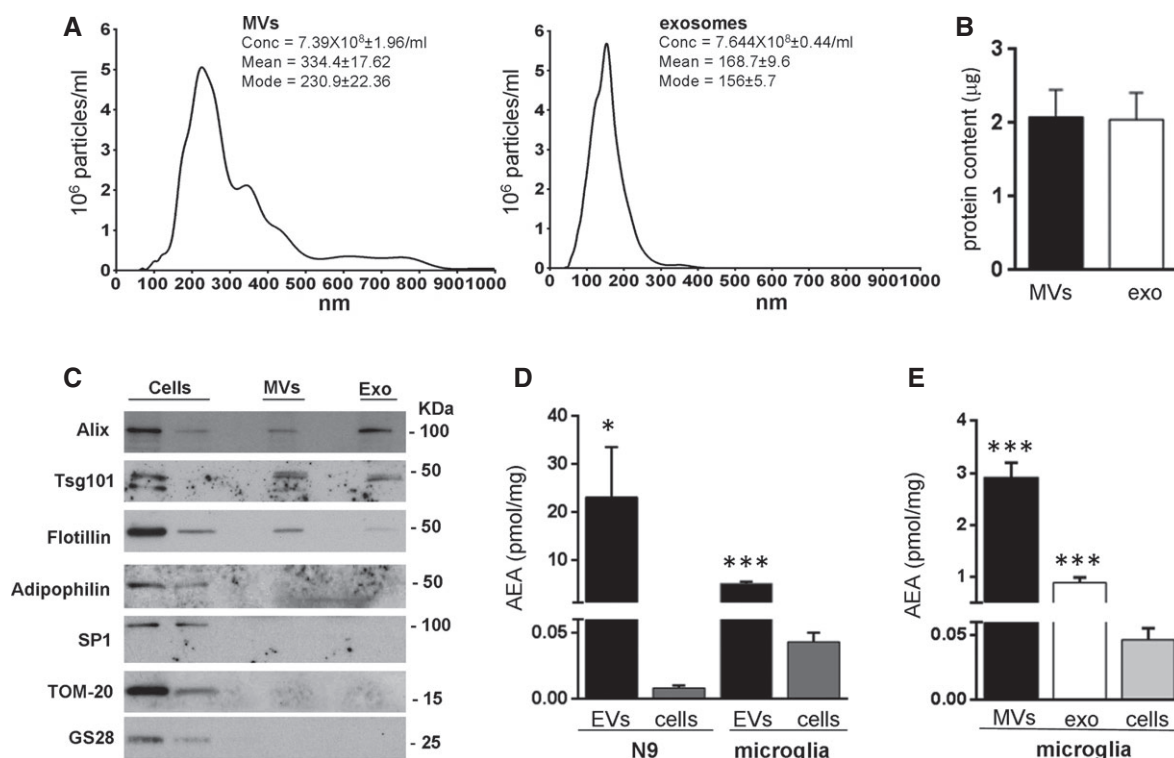


Figure 1. AEA is enriched in microglial EVs.

A Size profile of MV- (left) and exosome- (right) enriched fractions, pelleted from 1×10^6 primary microglia and resuspended in 400 µl of 0.1-µm-filtered sterile KRH. Normalized EV concentration: MVs = 1 ± 0.05 , exosomes = 1.2 ± 0.2 ($N = 3$).
 B Protein amount of MVs and exosomes yielded by 1×10^6 primary microglia ($N = 14$).
 C Western blot analysis of MV- and exosome-enriched fractions produced from 1×10^7 primary microglia with the indicated antibodies. Note the absence of immunoreactivity for mitochondrial, Golgi and nuclear markers (TOM-20, GS28, SP1). The first two lanes show lysates from 2×10^5 and 0.4×10^5 cells.
 D Histograms show AEA content in EVs versus corresponding donor N9 cells ($N = 2$), or primary rat microglia ($N = 4$). Student's *t*-test, * $P < 0.05$, *** $P < 0.001$.
 E AEA content in MVs and exosomes versus corresponding donor primary microglia. One-way ANOVA, $P < 0.0001$, Bonferroni *t*-test for comparison among groups, *** $P < 0.0001$, $N = 3$.

Data information: Data are presented as mean \pm SE.
 Source data are available online for this figure.

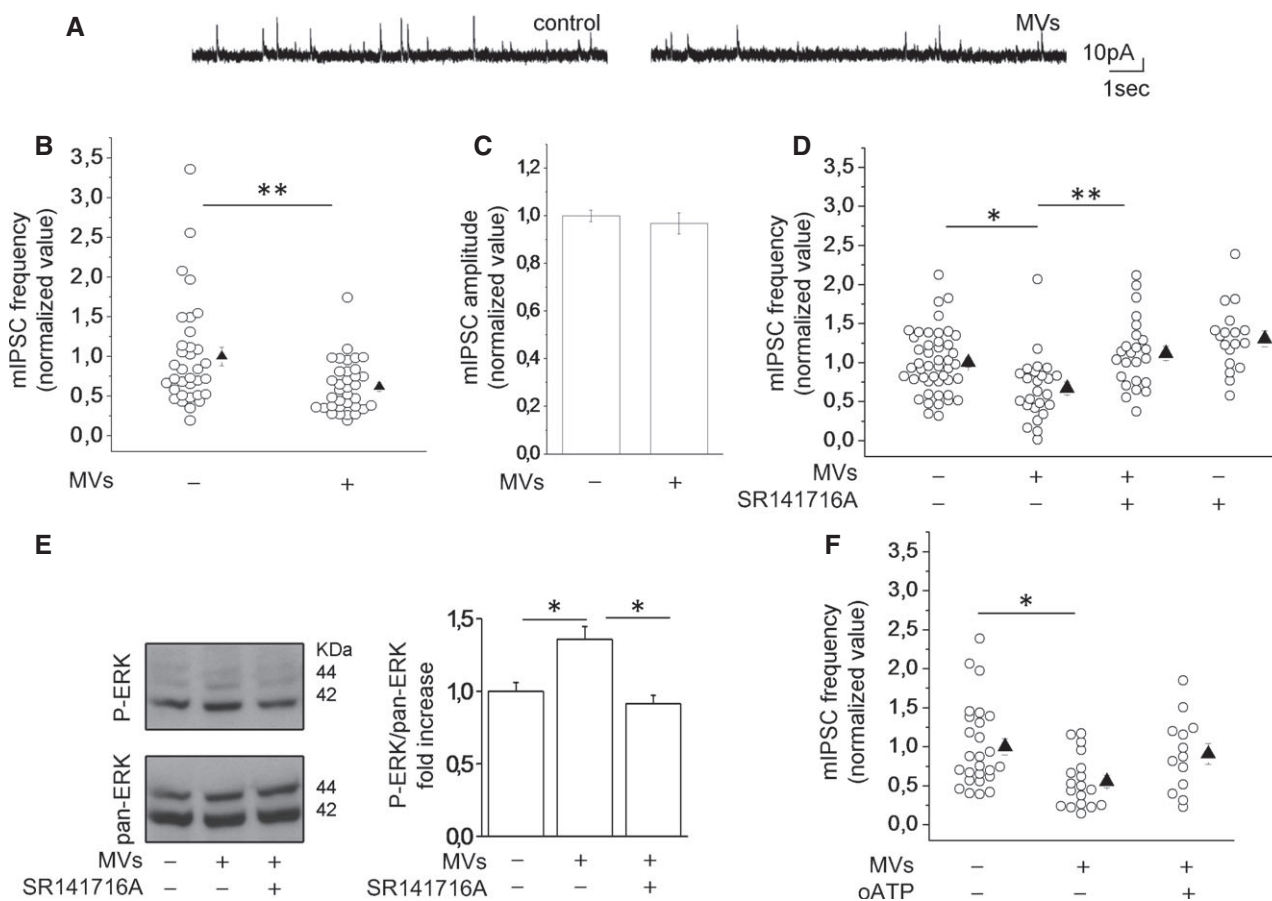


Figure 2. Effect of microglial MVs on GABAergic neurotransmission and ERK phosphorylation.

A Representative traces of mIPSCs from 16 to 17 DIV control neurons and neurons pre-exposed to MVs derived from primary microglia (10,000 g pellet) for 40–45 min.

B, C Changes of mIPSC frequency (B, normalized data, Mann–Whitney rank-sum test, ** $P = 0.004$; $N = 5$) but not amplitude (C, normalized data, Mann–Whitney rank-sum test, $P = 0.428$, Ctr $n = 35$ cells, MVs $n = 35$ cells; $N = 5$) evoked by MVs.

D Normalized mIPSC frequency changes evoked by MVs in neurons pre-treated for 15 min with the CB₁ antagonist SR141716A (1 μ M). One-way ANOVA, $P < 0.001$, Bonferroni t -test for comparison among groups, * $P < 0.05$, ** $P < 0.01$, $N = 5$.

E Western blot analysis showing changes of ERK-protein phosphorylation 5 min after exposure to microglial MVs, in control neurons or neurons pre-treated with 1 μ M SR141716A. Kruskal–Wallis ANOVA, $P = 0.002$, Dunn's test for comparison among groups, * $P < 0.05$, $N = 3$.

F mIPSC frequency from control neurons and neurons exposed to the 10,000 g pellet, collected from medium conditioned by microglia in the presence of the P2X₇ antagonist oATP. Normalized data, Kruskal–Wallis ANOVA, $P = 0.006$, Dunn's test for comparison among groups, * $P < 0.05$, $N = 3$.

Data information: Data are presented as mean \pm SE.

Source data are available online for this figure.

revealed higher AEA levels in MVs relative to exosomes (Fig 1E). Thus, MVs shed from the surface of primary microglia were used in all subsequent experiments.

Microglial MVs activate CB₁ on cultured inhibitory neurons and suppress spontaneous release of GABA

Suppression of GABA release from hippocampal interneurons by activation of presynaptic CB₁ is a widely recognized function of AEA [3,5,22], which is often used to assay its biological activity. Therefore, we used patch-clamp recording of inhibitory neurotransmission in primary hippocampal cultures as a sensitive readout for vesicular AEA activity.

Cultured hippocampal neurons maintain many aspects of *in vivo* physiology [23], including the highest density of functional CB₁ on

GABAergic axon terminals [22,24–26]. Consistent with the CB₁ receptor localization at GABAergic synapses (Supplementary Fig S1A), a clear suppression of miniature inhibitory postsynaptic currents (mIPSCs) was induced by acute application of CB₁ agonist WIN55,212-2, used at the effective concentration of 1 μ M [27,28]. Instead, no major alterations of miniature EPSC (mEPSC) frequency were detectable (Supplementary Fig S1B).

Exposure of hippocampal neurons to microglia-derived MVs (1.2 μ g/ml, 10,000 g ectosome-enriched pellet, produced at a microglia-to-neuron ratio of 2:1, 40–45 min) induced a significant decrease in mIPSC frequency (Fig 2A and B), mimicking the suppression of spontaneous inhibitory transmission evoked by the CB₁ agonist WIN55,212-2 (Supplementary Fig S1B). The decrease in mIPSC frequency was not associated with amplitude changes (Fig 2C), consistent with a presynaptic effect. The involvement of

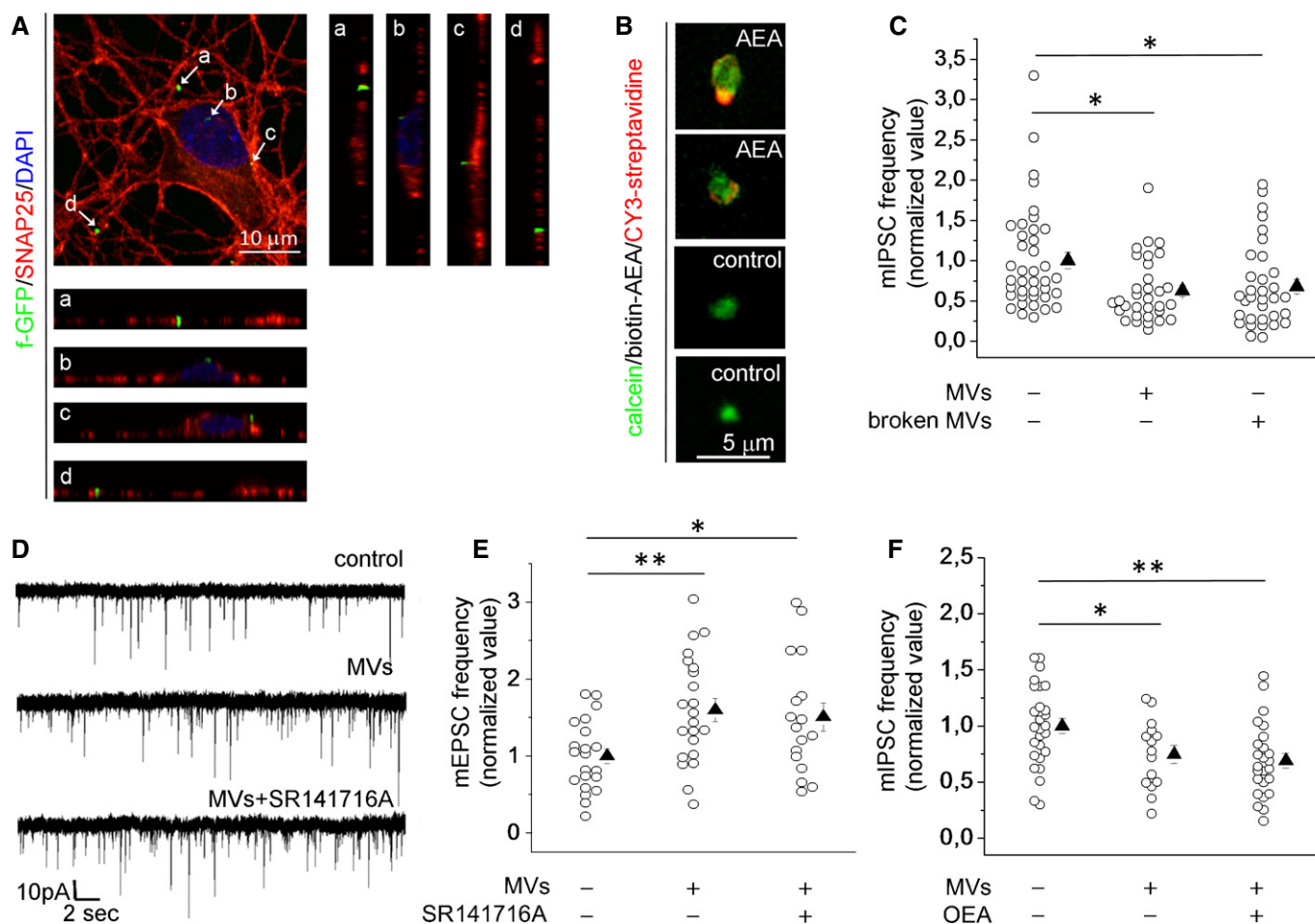


Figure 3. Microglial MVs inhibit mIPSC frequency and potentiate mEPSC frequency by distinct molecular pathways.

- A** MVs shed from primary cells transfected with f-GFP, were isolated at 10,000 g and incubated with hippocampal neurons for 3 h. Cultures were then fixed and stained for the plasma membrane protein SNAP-25 (red), GFP (green) and the nuclear marker DAPI (blue). The maximal projection image shows a clear association of f-GFP-labeled MVs with the neuronal surface, further revealed by the x-y projections (a–d).
- B** Examples of calcein-storing MVs (green) showing surface AEA staining (red) after incubation with biotin-AEA followed by Cy3-streptavidin. The presence of red aggregates suggests non-homogeneous AEA distribution on MV surface. No fluorescent signal is detectable on the surface MVs directly incubated with Cy3-streptavidin (control).
- C** Normalized mIPSC frequency changes evoked by MVs broken by hypo-osmotic stress. Kruskal–Wallis ANOVA, $P = 0.003$, Dunn's test for comparison among groups, * $P < 0.05$, $N = 3$. Data are presented as mean \pm SE.
- D** Representative traces of mEPSCs from control neurons and neurons exposed to MVs in the presence or in the absence of CB₁ antagonist SR141716A (1 μ M).
- E** Changes of mEPSC frequency evoked by MVs in control neurons and neurons pre-treated for 15 min with SR141716A (1 μ M). Normalized data, one-way ANOVA, $P = 0.007$, Holm–Sidak's test for comparison among groups, * $P < 0.05$, ** $P < 0.01$, $N = 4$. Data are presented as mean \pm SE.
- F** Changes of mIPSC frequency evoked by microglial MVs in hippocampal neurons pre-treated or not with the inhibitor of sphingosine metabolism OEA (37 μ M). Normalized data, one-way ANOVA, $P = 0.003$, Holm–Sidak's test for comparison among groups, * $P < 0.05$, ** $P < 0.01$, $N = 5$. Data are presented as mean \pm SE.

Source data are available online for this figure.

vesicular eCBs in this phenomenon was inferred from the ability of the selective CB₁ antagonist SR141716A, used at the effective concentration of 1 μ M [27,28], to completely block the reduction of mIPSC frequency evoked by MVs (Fig 2D). No significant changes of mIPSC frequency occurred after culture incubation with SR141716A, in the absence of MVs.

Activation of CB₁ by MV-associated AEA translated into downstream signaling, consisting in a significant phosphorylation of ERK, a mitogen-activated protein kinase regulated by CB₁ [29]. ERK phosphorylation was detected 5 min after MV application and was

completely inhibited by co-administration of the CB₁ antagonist SR141716A at 1 μ M (Fig 2E).

To rule out that AEA may be released by microglia through non-vesicular pathways, such as lipid droplets or micelles that may co-purify with MVs, we inhibited ATP-induced MV secretion with the P2X₇ receptor antagonist oATP (100 μ M), as previously described [17,19]. Figure 2F shows that the 10,000 g pellet collected from the supernatant under blockade of ATP-induced MV release was unable to decrease mIPSC frequency. This finding indicates that MVs actually account for the biological activity of

the 10,000 g pellet. Consistently, Western blot analysis for the specific lipid droplet marker adipophilin [30] ruled out any contamination by lipid accumulations in the MV-enriched fraction (Fig 1C).

These findings outline a crucial role for AEA produced by microglia and released extracellularly in association with MVs in the modulation of inhibitory transmission.

MVs carry AEA on their surface

Activation of presynaptic CB₁ by microglial ectosomes requires contact between MVs and recipient neurons. Accordingly, confocal analysis of neurons exposed for 3 h to f-GFP-labeled MVs, extensively washed and stained with the plasma membrane-associated protein SNAP-25, revealed binding of MVs to the neuronal surface. MV binding occurred along neurites but also in the somatodendritic compartment and was not followed by MV internalization as indicated by the *x-y* projections (Fig 3A).

To activate presynaptic CB₁, vesicular eCBs should be located on the MV surface. Consistently, incubation of MVs with a biotinylated analog of AEA (5 μ M), designed for AEA visualization [31], followed by incubation with Cy3-streptavidin, induced positive labeling of MVs (Fig 3B), suggesting affinity of AEA for the MV membrane.

The next step was to investigate whether modulation of GABAergic transmission requires intact MVs. To this end, neurons were incubated with broken MVs, destroyed by hypo-osmotic stress and re-pelleted to remove their luminal cargo. Figure 3C shows that broken MVs retained the capability to decrease mIPSC frequency, suggesting that MV integrity is not required for AEA-mediated activation of presynaptic CB₁ (Fig 3C).

Microglial MVs induce alteration of excitation/inhibition balance

We recently showed that microglial MVs enhance glutamatergic transmission through promotion of ceramide and sphingosine synthesis in excitatory neurons [32]. To clarify whether the suppression of GABAergic tone described above might contribute to the enhancement of excitatory transmission evoked by microglial MVs, we investigated how MVs may impact mEPSC frequency in the presence of the CB₁ antagonist SR141716A. Figure 3D and E shows that microglial MVs induced a significant increase in mEPSC frequency, which was not prevented by SR141716A. Additionally, the latter substance was ineffective when used alone (normalized values: Ctr = 1.000 ± 0.107 $n = 18$; SR141716A-treated = 1.151 ± 0.151 $n = 13$ cells, $N = 3$; Student's *t*-test, $P = 0.407$). These data indicated that MVs directly potentiate excitatory transmission, independently of the modulation of the inhibitory tone. We also asked whether potentiation of excitatory transmission could cross-affect inhibitory transmission. Figure 3F shows that neuron pretreatment with the inhibitor of sphingosine metabolism *N*-oleoyl-ethanolamine (OEA, 37 μ M), which is known to prevent presynaptic stimulation of excitatory transmission evoked by MVs [32], did not prevent the decrease in mIPSC frequency caused by MVs. The latter substance was ineffective when used alone (normalized values: Ctr = 1.000 ± 0.117 $n = 22$ cells; OEA-treated = 1.186 ± 0.173 $n = 19$ cells, $N = 3$; Mann-Whitney rank-sum test, $P = 0.539$). Incidentally, it should be recalled that OEA is an endogenous peroxisome proliferator-activated receptor- α and GPR119 agonist, which acts independently of CB₁ [33].

Discussion

Different hypotheses have been proposed to explain the transport of AEA in and out of the cell, and nowadays the evergrowing complexity of eCB trafficking makes this topic a hot spot in this research area [34,35]. AEA translocation across the cell plasma membrane might be ascribed to a protein carrier [34] and, once taken up, AEA might move intracellularly via fatty acid binding proteins, heat shock proteins or albumin [14]. Another specific intracellular transporter of AEA, termed FAAH-like AEA transporter (FLAT), has been identified [36], but its actual role as an AEA carrier has been recently questioned [37]. Neosynthesized AEA could be also released into the extracellular fluid and reach its targets bound to circulating proteins, with lipocalins and albumin being good candidate [38]. Here, we report for the first time that the eCB AEA is present in EVs, both MVs and exosomes, produced by microglia and remarkably enriched in these vesicles as compared to parental cells. This evidence identifies EVs as an additional vehicle for the transport of hydrophobic compounds like eCBs across the nervous system. Compared to previous studies, we detected a lower absolute concentration of AEA in cultured microglia [9,10]. Yet, eCBs are labile substances, difficult to measure, and their levels are known to vary depending on the experimental conditions. We also demonstrate that vesicular eCBs are biologically active, as MVs carrying AEA on their surface activate CB₁ and its downstream signaling in cultured GABAergic neurons, and inhibit mIPSC frequency.

eCBs are essential signaling molecules that regulate axon guidance and neuritogenesis in GABAergic interneurons during the critical period of postsynaptic target selection [39]. In this context, microglia are known to modulate neural circuit formation and function in the developing brain by the release of active molecules [40]. Our findings, by providing evidence for the activation of CB₁ in GABAergic neurons by microglia-derived MVs, suggest that microglia may crucially regulate positioning and circuit formation of CB₁-expressing interneurons through secretion of AEA-storing EVs during brain development. Notably, EVs produced by microglia also carry the hydrophobic lipid-modified protein Wnt3a [15], which acts as a morphogen during development [41]. Thus, it is possible that microglia packages into EVs different hydrophobic secreted molecules, which are involved in the establishment of brain circuits. Additional experiments are required to explore this hypothesis.

Our data also suggest that microglia-derived MVs may affect excitation/inhibition balance in the adult nervous system. Indeed, the decrease in GABAergic transmission evoked by microglial MVs suggests that ectosome release, which dramatically increases upon brain inflammation [20,42], may function as a mechanism by which microglia participate in excitatory phenomena [43–45]. Consistently with this possibility, we have recently shown that microglial MVs enhance glutamatergic transmission *in vitro* and acutely increase the excitation/inhibition balance in the rat visual cortex [32]. We attributed these effects to the ability of microglial MVs to promote in excitatory neurons synthesis of sphingosine [32], a bioactive sphingolipid that enhances presynaptic release probability [46]. Additionally, we now show that microglial MVs directly suppress the GABAergic tone through CB₁ activation on interneurons, thereby contributing to alteration of excitation/inhibition balance.

Collectively, these findings indicate that microglial MVs transport various hydrophobic mediators, that is, eCBs and yet unidentified

lipids which promote sphingosine metabolism [32], to mediate opposite but synergic actions on inhibitory and excitatory presynaptic terminals. This hypothesis is consistent with the general view that multiple bioactive molecules are sorted into EVs and that distinct messages may be delivered by EVs of same origin according to the types of recipient cells.

Materials and Methods

Animals

All the experimental procedures followed the guidelines established by the European Legislation (Directive 2010/63/EU) and the Italian Legislation (L.D. no 26/2014).

Cell cultures and treatments

N9 microglia and primary microglia from P1 rats were obtained and maintained as previously described [32]. Hippocampal neurons were established from E18 fetal rats (see Supplementary Materials and Methods).

Isolation of MVs, exosomes or mixed EVs

Conditioned medium was pre-cleared from cells and debris. MVs and exosomes were then pelleted at 10,000 g and 100,000 g, respectively, while mixed EVs were centrifugated at 100,000 g (see Supplementary Materials and Methods).

Nanosight analysis

The number and dimension of MVs and exosomes was assessed with Nanosight LM10-HS system (see Supplementary Materials and Methods).

Western blotting

Cellular or vesicular extracts were separated by electrophoresis, blotted and probed using antibodies against ERK 1/2 and P-ERK 1/2, Tsg101, alix, SP1, TOM-20, adipophilin and GS28 (see Supplementary Materials and Methods). Photographic development was by chemiluminescence and quantification by ImageJ software.

Endogenous levels of AEA and 2-AG

Lipids were extracted with chloroform/methanol (2:1, v/v). The organic phase was dried and analyzed by LC-ESI-MS. Quantitative analysis was performed as previously reported [47] (see Supplementary Materials and Methods).

Immunofluorescence staining

Immunofluorescence was performed using the following antibodies: rabbit anti-CB₁, guinea pig anti-vGLUT1, human anti-GAD, mouse anti-SNAP-25 and goat anti-GFP. Secondary antibodies were conjugated with Alexa-488, Alexa-555 or Alexa-633 fluorophores. To visualize AEA, calcein-loaded MVs were incubated with 5 μ M

biotin-AEA for 10 min, washed and incubated with Cy3-streptavidin. After further washing, MVs were resuspended in KRH, spotted on glass coverslip and observed with microscope (see Supplementary Materials and Methods).

Electrophysiological recordings

Whole-cell patch-clamp recordings were performed using a Multi-Clamp 700A amplifier and pCLAMP 10 Software. Signals were sampled at 10 kHz and filtered to 4 kHz. mIPSCs and mEPSCs were recorded in the presence of tetrodotoxin (1 μ M). (see Supplementary Materials and Methods).

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

MG, NB, LR and IP performed the experiments and analyzed the data. FA performed the experiments and discussed the data. LC analyzed the data. MM discussed the data and wrote the manuscript. MM and CV designed the study, discussed the data and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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